AMERICAN SOCIETY FOR MICROBIOLOGY | Antimicrobial Agents and Chemotherapy®

EPIDEMIOLOGY AND SURVEILLANCE

Genome Analysis of *Klebsiella oxytoca* Complex for Antimicrobial Resistance and Virulence Genes

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ABSTRACT *Klebsiella oxytoca* complex comprises nine closely related species causing human infections. We curated genomes labeled *Klebsiella* (n = 14,256) in GenBank and identified 588 belonging to the complex, which were examined for precise species, sequence types, K- and O-antigen types, and virulence and antimicrobial resistance genes. The complex and *Klebsiella pneumoniae* share many K- and O-antigen types. Of the complex, *K. oxytoca* and *Klebsiella michiganensis* appear to carry more virulence genes and be more commonly associated with human infections.

KEYWORDS resistance, *Klebsiella oxytoca*, virulence, taxonomy, *Klebsiella*, antimicrobial resistance

Klebsiella oxytoca and eight closely related species—*Klebsiella grimontii*, *Klebsiella huaxiensis*, *Klebsiella michiganensis*, *Klebsiella pasteurii*, *Klebsiella spallanzanii*, and three new unnamed ones (taxons 1, 2, and 3)—comprise a complex, that are difficult, if not impossible, to be reliably differentiated by phenotypic characteristics (1). *K. oxytoca* complex is a member of the normal gut microflora (2, 3) and also an important human pathogen causing antibiotic-associated hemorrhagic colitis (AAHC) and a variety of other infections (4–7). Although several studies have addressed antimicrobial resistance and virulence (8, 9), there are large knowledge gaps in our understanding clinically relevant aspects of the complex, such as the prevalence or the proportion of each species of *K. oxytoca* complex in clinical samples, the virulence factors other than cytotoxins causing AAHC, and the strain clonal background (1). Genome sequences available in the public domain may provide useful information to complement literature for understanding these aspects. We therefore examined all available genomes of *K. oxytoca* complex in GenBank and generated a curated database for reference.

Precise species identification for all *Klebsiella* genomes identified 588 genomes of *K. oxytoca* complex. We used txid570 [Organism:exp] AND "latest" [filter] to search NCBI and found 14,256 genome sequences labeled as *Klebsiella* (accessed by 1 January 2021). We discarded 855 genomes for any of the following reasons: (i) assemblies are of low quality as defined by the NCBI, such as being labeled as "many frameshifted protein," "fragmented assembly," "genome length too small/large," etc. (n = 472); (ii) assemblies are duplicated, as they share the same BioSample (n = 225); (iii) assemblies are labeled as "contaminated" (n = 118); (iv) assemblies are labeled as "derived from metagenome" (n = 36); (v) assemblies belong to genetically modified organisms (GMO; n = 3); or (vi) assemblies are derived from an uncultured source (n = 1). Therefore, 13,401 genome sequences labeled as *Klebsiella* (see Data Set S1 in the supplemental material) were included. We therefore determined their precise species assignations using the average nucleotide identity (ANI) based on BLAST to compare with type

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The authors declare no conflict of interest.

Received 13 November 2021
Returned for modification 3 December 2021
Accepted 5 January 2022

Accepted manuscript posted online 10 January 2022
Published 15 March 2022

strains of *Klebsiella* and *Raoultella* species (see Table S1), as described previously with a ≥95 to 96% ANI as the species cutoff (10). For genomes with a <97% ANI comparing with any type strains of the *Enterobacteriaceae*, *in silico* DNA-DNA hybridization (isDDH) was further determined between the genome and that of the closest (sharing the highest ANI value) type strain to confirm or to determine the species status with a ≥70.0% isDDH as the species cutoff (11). Among the 13,401 genomes, 18 do not belong to the genus *Klebsiella*, and 55 are *Klebsiella* spp. but could not be assigned to a known species (see Table S2 and Data Set S1). Instead, the 55 strains could be assigned to two novel *Klebsiella* species named here taxon 4 (*n* = 16) and taxon 5 (*n* = 39) (see Table S2). Taxons 4 and 5 do not belong to *K. oxytoca* complex but are most closely related to *K. aerogenes* (95.92% ANI and 66.7% isDDH) and *K. pneumoniae* (95.87% ANI and 66.8% isDDH), respectively.

Among the 13,383 remaining *Klebsiella* genomes, there were only 588 (4.39%) belonging to *K. oxytoca* complex. The numbers of each species are as follows: *K. oxytoca* (*n* = 183), *K. michiganensis* (*n* = 233), *K. grimontii* (*n* = 128), *K. huaxiensis* (*n* = 3), *K. pasteurii* (*n* = 31), *K. spallanzanii* (*n* = 4), taxon 1 (*n* = 1), taxon 2 (*n* = 1), and taxon 3 (*n* = 4) (see Data Set S2). Species of *K. oxytoca* complex have similar genomic characteristics (genome size, GC content, and numbers of coding sequences and tRNA) with other *Klebsiella* species and *Raoultella* spp. (see Table S3).

To identify genes unique to the complex, a subset of genomes (*n* = 1,410), including 191 of *K. oxytoca* complex was retrieved from all 13,383 *Klebsiella* assemblies to adapt local computational resources using Assembly Dereplicator v0.1.0 (https://github.com/rrwick/Assembly-Dereplicator). Coding sequences annotated using Prokka v1.14.6 (12) were clustered using PIRATE v1.0.4 (13) to identify genes that present in 99% *K. oxytoca* complex genomes but absent from all other *Klebsiella* genomes. These candidate genes were then challenged with the entire data set of 13,383 genomes using BLASTP algorithm, and hits with coverage or identity of <80% were discarded. As such, 35 genes unique to *K. oxytoca* complex were identified. Among these, 19 genes, including the intrinsic bla_{OXY} (see Data Set S3), could be assigned with a known KEGG orthology number of 11 KEGG pathways using KofamKOALA v2021-11-01 (14), KEGG mapper v5.0 (15), and KOBAS v3.0 (16). Notably, a type II secretion system to secrete pullulanase, a lipoprotein allowing growth on branched maltodextrin polymers (17), was the solely over-represented protein among all pathways (see Data Set S3, corrected *P* value of <0.001 [as determined by the Fisher exact test and Benjamini-Hochberg *P* value adjustment]). The contribution of these unique genes to the evolution of *K. oxytoca* complex warrants further studies.

***K. oxytoca* and *K. michiganensis* are the two main species associated with human infections.** The prevalence of each species of *K. oxytoca* complex in human colonization and infection is largely unknown since strains called *K. oxytoca* in most clinical studies were not subjected to precise species identification (1). Of the 588 genomes, 154 were recovered from human samples other than feces and rectal swabs as we focused on extraintestinal infections. The 154 genomes belong to *K. oxytoca* (*n* = 96), *K. michiganensis* (*n* = 46), *K. grimontii* (*n* = 5), *K. pasteurii* (*n* = 4), *K. spallanzanii* (*n* = 2), and *K. huaxiensis* (*n* = 1) (see Data Set S2). *K. oxytoca* and *K. michiganensis* appear to be the two main species of the complex associated with human extraintestinal infections. However, genome sequencing is usually highly biased, and well-designed surveillance studies are therefore required to demonstrate the true distribution of each species within *K. oxytoca* complex.

Several sequence types were relatively common. The 588 genomes can be assigned to 227 sequence types (STs), including 121 known STs and 116 new STs (assigned as N1 to N116 here) by query the multilocus sequence typing database (https://pubmlst.org/organisms/klebsiella-oxytoca) (see Data Set S2 and Table S4 for the number of genomes belonging to each ST). This is consistent with previous reports (18, 19), suggesting that very diverse clonal background of the complex. Only six STs have at least 10 genomes and have been seen in at least three countries, including ST2 (*gapA-infB-mdh-pgi-phoE-rpoB-tonB* allele profile, 1-2-2-1-2-1-2; *n* = 25, in Denmark, Spain, the UK, and the USA) and

ST176 (1-7-2-1-65-1-2; *n* = 12, in Australia, Canada, the UK, and the USA) of *K. oxytoca*, ST29 (3-4-15-8-18-6-11; *n* = 11, in Germany, South Africa, and the UK), ST85 (3-5-21-13-24-6-19; *n* = 16, in China, the UK, and the USA), and ST88 (3-8-24-33-20-6-23; *n* = 10, in Australia, the UK, and the USA) of *K. michiganensis*, and ST215 (21-6-14-10-46-11-6; *n* = 10, in China, the UK, and the USA) of *K. grimontii*. Single nucleotide polymorphisms (SNPs) between genomes of the same ST were called using Snippy v4.6.0 (https://github.com/tseemann/snippy) and were filtered to remove recombination using Gubbins v2.4.1 (20). No isolates of the aforementioned six STs in different countries belonged to the same clone based on SNP calling (see Data Set S4 for SNP numbers of these STs). This suggests that no international transmission of the same clone was identified at present, but these STs may need to be monitored to reveal their potential to become high-risk clones.

The K- and O-antigen types of *K. oxytoca* complex could overlap *K. pneumoniae*. The major surface antigens of *Klebsiella* are capsular polysaccharide (CPS; K antigen) and lipopolysaccharide (LPS; containing O antigen), which are common virulence factors of *K. pneumoniae* (21–24) but are less studied in *K. oxytoca* complex. We performed the serotyping of K and O antigens for the 588 genomes using Kleborate (25). Of 588 genomes, known K-antigen types can only be assigned to 194 (33.0%) genomes, which belong to *K. grimontii*, *K. michiganensis*, *K. oxytoca*, or *K. pasteurii*, while the remaining 394 genomes contain K-antigen loci of novel K types. A total of 14 known K types (K26, K29, K41, K43, K66, K70, K74, K102, K109, K145, K152, K157, K164, and K169) were identified, among which K74 (9.2%, 54/588), K29 (3.4%, 20/588), and K43 (2.9%, 17/588) were the most common (see Data Set S2). K102, K109, K145, K152, K157, K164, and K169 have not yet been reported. All of the K types identified in *K. oxytoca* complex other than K157 and K164 have also been seen in *K. pneumoniae* (25–31). Currently, there are no published reports of the O antigen in *K. oxytoca* complex (1). Known O-antigen types could be assigned to the vast majority (96.1%, 565/588) of the genomes across all species of *K. oxytoca* complex, and 7 O antigen types (O1, O5, OL104, O2, O3/O3a, O3b, and O4) were identified, with O1 (52%, 306/588), O5 (14.3%, 86/588), and OL104 (11.9%, 70/588) as the most common types. All of the O-antigen types have also been reported in *K. pneumoniae* (24, 32, 33).

***K. oxytoca* complex carries multiple virulence factors seen in *K. pneumoniae*.** *K. oxytoca* is well known for causing AAHC, which is due to the production of cytotoxins tilimycin and tilivalline (a derivative of tilimycin) (34, 35). Among the 9 species of *K. oxytoca* complex, the tilimycin gene cluster is absent from *K. huaxiensis*, *K. spallanzanii*, and taxons 1, 2, and 3 (Table 1), suggesting that these species may not cause AAHC. Other than the AAHC-associated cytotoxins, virulence factors of *K. oxytoca* complex remain largely unknown. As such, in addition to examining the presence of the tilimycin gene cluster, we also screened the 588 genomes for known virulence factors of *K. pneumoniae* (*n* = 80; https://bigsdb.pasteur.fr/cgi-bin/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef&page=downloadAlleles&scheme_id=4&render=1) (36) using BLASTn with a threshold of 90% for both coverage and identity. A total of 72 virulence genes were detected (see Table 1 and Data Set S2). These genes encode adhesins or biofilm formation (*fimA*, *fimH*, KP1, *mrkABCDFHJ*, and *pilQ*), allantoin utilization (*allABCDRS*, *fdrA*, *gcl*, *glxKR*, *hyi*, *ybbWY*, and *ylbF*), capsules (*matB*), microcin (*mceABCDEHGIJ*), iron acquisition (*clbA-R*, *fyuA*, *iroN*, *irp*, *iutA*, *kfuABC*, and *ybtAEPQTUX*), and urease (*ureA*). Among these genes, *fimA*, *fimH*, *iutA*, *matB*, *mrkABCDFHJ*, *pilQ*, and *ureA* were present in almost all of the 588 genomes (Table 1). Of note, *rmpA* and *rmpA2* (regulators of the hypermucoviscous phenotype) were not found in *K. oxytoca* complex. Among the 9 species, there appeared to be two patterns of the distribution of virulence genes. The four species most often seen in clinical samples in the present data set (see Data Set S2)—*K. michiganensis*, *K. oxytoca*, *K. grimontii*, and *K. pasteurii*—carried ≥45 virulence genes, with *K. michiganensis* carrying all 72 genes, followed by *K. oxytoca* (carrying 51 genes). In contrast, the other five species had 30 to 35 genes (Table 1). Iron acquisition genes *fyuA*, *iroN*, *irp*, and *ybtAEPQTUX* were enriched in the genomes of *K. michiganensis*, *K. oxytoca*, *K. grimontii*, and *K. pasteurii* but absent from those of the other five species (Fig. 1 and Table 1). Another iron acquisition gene, *iroN*, was present in most (75.9%, 177/233) *K. michiganensis* samples but was only seen in only a minority of *K. oxytoca* (21.3%, 39/183) and *K. grimontii* (11.7%, 15/128)

TABLE 1 Virulence factors in genomes of *K. oxytoca* complex (*n* = 588)

	Klebsiella[b]						Taxon			
Virulence factor(s)	***grimo***	***huaxi***	***michi***	***oxyto***	***paste***	***spall***	**1**	**2**	**3**	**Frequency (%)**
No. of genomes	128	3	233	183	31	4	1	1	4	
No. of genes[a]	45	33	72	51	45	35	30	33	33	
allA, *allS*	128	3	42	183	31	4	1	1	4	67.5
allB	111	3	42	183	31	4	1	1	4	64.6
allC	127	3	42	183	31	4	1	1	4	67.3
allD	127	3	42	182	31	4	1	1	4	67.2
allR	128	3	42	182	30	4	1	1	4	67.2
arcC	128	3	42	182	31	4	1	1	4	67.3
clb genes[c]	0	0	2	0	0	0	0	0	0	0.3
fdrA	127	3	42	183	31	4	1	1	4	67.3
fimA	127	3	233	183	31	4	1	1	4	99.8
fimH	123	3	229	182	31	4	1	1	4	98.3
fyuA	72	0	158	175	31	0	0	0	0	74.1
gcl, *hyi*	128	3	42	183	31	4	1	1	4	67.5
glxK	128	3	42	181	31	4	1	1	4	67.2
glxR	128	3	41	183	31	4	1	1	4	67.3
iroN	15	3	177	39	0	3	1	1	4	41.3
irp1	70	0	158	172	31	0	0	0	0	73.3
irp2	69	0	158	175	29	0	0	0	0	73.3
iutA	128	3	233	182	31	4	1	1	4	99.8
kfuA	128	3	233	0	31	3	0	1	1	68.0
kfuB, *kfuC*	127	3	233	0	31	3	0	1	1	67.9
KP1_1364, *ylbF*	128	3	42	183	31	4	1	1	4	67.5
KP1_1371	127	3	42	183	31	4	1	1	4	67.3
matB	125	3	233	182	31	4	1	1	4	99.3
mceA, *mceB*, *mceE*	0	0	1	1	0	0	0	0	0	0.3
mceC	0	0	1	29	0	2	0	0	0	5.4
mceD, *mceJ*	0	0	1	28	0	0	0	0	0	4.9
mceG	0	0	2	29	0	0	0	0	0	5.3
mceH	0	0	2	29	2	0	0	0	0	5.6
mceI	0	0	1	28	0	2	0	0	0	5.3
mrkA	125	3	232	180	31	4	1	1	4	98.8
mrkB	126	3	229	180	31	4	1	1	4	98.5
mrkC	119	3	225	180	31	4	1	1	4	96.6
mrkD	125	3	231	180	31	4	1	1	4	98.6
mrkF	125	3	228	180	31	4	1	1	4	98.1
mrkH	127	3	231	182	30	4	1	1	4	99.1
mrkJ	127	3	230	178	31	4	1	1	4	98.5
pilQ	128	3	233	182	30	4	1	1	4	99.7
ureA	128	3	233	182	31	4	1	1	4	99.8
ybbW	127	3	42	181	31	4	1	1	4	67.0
ybbY	128	3	41	179	31	4	1	1	4	66.7
ybtA	70	0	159	175	31	0	0	0	0	74.0
ybtE	72	0	158	175	31	0	0	0	0	74.1
ybtP	68	0	159	172	31	0	0	0	0	73.1
ybtQ	70	0	159	176	31	0	0	0	0	74.1
ybtS	68	0	159	175	31	0	0	0	0	73.6
ybtT	72	0	157	175	31	0	0	0	0	74.0
ybtU	71	0	157	175	31	0	0	0	0	73.8
ybtX	71	0	158	176	30	0	0	0	0	74.0
Tilimycin gene cluster	110	0	34	141	26	0	0	0	0	52.9

[a]Genes with an identical distribution pattern are clustered together. The number of genes does not contain the tilimycin gene cluster.
[b]Species: *grimo*, *grimontii*; *huaxi*, *huaxiensis*; *michi*, *michiganensis*; *oxyto*, *oxytoca*; *paste*, *pasteurii*; *spall*, *spallanzanii*.
[c]The *clb* cluster contains 18 genes (*clbA*, *clbB*, *clbC*, *clbD*, *clbE*, *clbF*, *clbG*, *clbH*, *clbI*, *clbJ*, *clbK*, *clbL*, *clbM*, *clbN*, *clbO*, *clbP*, *clbQ*, and *clbR*). All 18 genes have an identical distribution pattern.

and was absent from *K. pasteurii* (Table 1). Although the distribution of virulence genes may provide clues to understand the clinical significance of individual species of *K. oxytoca* complex, the association of these genes with pathogenesis in the complex remains to be determined.

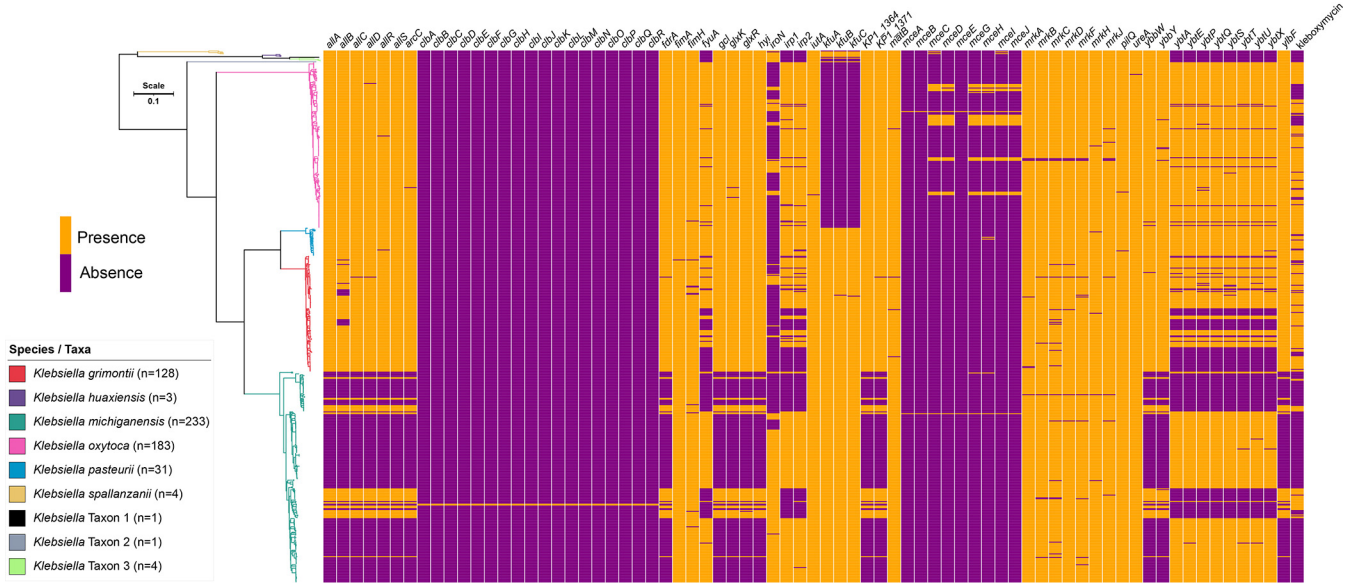

FIG 1 Phylogenomic tree of 588 genomes of *K. oxytoca* complex and distribution of virulence factors. The core genes of the *K. oxytoca* complex (n = 588) were identified and concatenated using PIRATE v.1.0.4 (13) with default settings, followed by the phylogenetic tree inference using IQ-TREE v2.1.3 (39) under GTR+G+ASC model with a 1,000-bootstrap test. Tree was then visualized and annotated using web-based tools Phandango v1.3.0 (40) and iTOL v6.4.2 (41).

***K. oxytoca* complex has a large number of acquired antimicrobial resistance genes.** *K. oxytoca* complex carries intrinsic β-lactamase-encoding bla_{OXY} and fosfomycin resistance gene *fosA* (1, 37). Species other than *K. huaxiensis*, *K. spallanzanii*, taxon 1, and taxon 3 also have intrinsic low-level quinolone resistance genes *oqxA-oqxB* (1). A variety of acquired genes mediating resistance to β-lactams (e.g., penicillins, cephalosporins, and carbapenems), aminoglycosides, colistin, chloramphenicol, macrolides, quinolones, rifampicin, sulfonamides, tetracyclines, and trimethoprim have been reported in literature (38) and have been summarized previously (1). By analyzing the 588 genomes, many antimicrobial resistance genes that have not been reported in literature were identified using ResFinder (http://genomicepidemiology.org/) (Table 2), highlighting the large defense arsenal in the complex. Among the 588 genomes, 109 contain one or more carbapenemase-encoding genes, including bla_{GES-5}, $bla_{IMP-4,\ -13,\ -29,\ -38}$, $bla_{KPC-2,\ -3}$, $bla_{NDM-1,\ -5}$, $bla_{OXA-48,\ -181}$, and bla_{VIM-1} (Table 2), which are commonly carried by plasmids (1). The most common one was bla_{KPC-2}, present in 63 genomes. However, there was no single ST that carried a carbapenemase gene and was present in at least three countries (see Data Set S2).

TABLE 2 Acquired antimicrobial resistance genes in genomes of *K. oxytoca* complex (n = 588)

Antimicrobial type	Acquired antimicrobial resistance gene(s)[a]
Carbapenems	bla_{GES-5}, $bla_{IMP-4,\ -13,\ -29,\ -38}$, $bla_{KPC-2,\ -3}$, $bla_{NDM-1,\ -4,\ -5}$, $bla_{OXA-48,\ -181}$, bla_{VIM-1}
Other β-lactams[b]	bla_{ACC-1}, $bla_{CARB-2,\ -12}$, bla_{CMY-6}, $bla_{CTX-M-1,\ -3,\ -8,\ -14,\ -15,\ -62}$, bla_{DHA-1}, bla_{FOX-5}, bla_{LAP-2}, $bla_{OXA-1,\ -2,\ -9,\ -10,\ -101}$, bla_{SCO-1}, bla_{SFO-1}, $bla_{SHV-2,\ -5,\ -7,\ -12,\ -14,\ -30}$, $bla_{TEM-1,\ -3,\ -26,\ -116}$, bla_{TLA-3}
Colistin	*mcr-9*
Aminoglycosides	*aac(2′)-IIa*, *aac(3)-Ia*, *-IId*, *-IIe*, *-IIg*, *-IVa*, *aac(6′)-30*, *-Ib*, *-Ib′*, *-Ib3*, *-Ib4*, *-Ib*-cr, *-Ib*-cr5, *-IIa*, *-IIc*, *aadA1*, *aadA*2, *aadA4*, *aadA5*, *aadA10*, *aadA11*, *aadA13*, *aadA16*, *ant(2″)-Ia*, *aph(3′)-Ia*, *-IIa*, *-VI*, *-XV*, *aph(3″)-Ib*, *aph(4)-Ia*, *aph(6)-Id*, *aphA16*, *armA*, *rmtC*, *sat2*
Quinolones	*qnrA1*, *qnrB1*, *qnrB2*, *qnrB4*, *qnrB6*, *qnrB19*, *qnrS1*
Sulfonamides	*sul1*, *sul2*, *sul3*
Trimethoprim	*dfrA1*, *dfrA3b*, *dfrA10*, *dfrA12*, *dfrA14*, *dfrA15*, *dfrA17*, *dfrA19*, *dfrA21*, *dfrA27*, *dfrA32*, *dfrB1*
Chloramphenicol	*catA1*, *catA2*, *catB2*, *catB3*, *catB8*, *catB11*, *cmlA1*, *cmlA4*, *cmlA5*, *cmlA10*, *cmx*, *floR*
Rifampicin	*arr-2*, *arr-3*
Tetracyclines	*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(M), *tet*(34), *tet*(39)
Macrolides	*ere*(A), *mph*(A), *mph*(E), *msr*(E)

[a]Resistance genes that have not been reported in the literature (1) but are identified in genomes available in the NCBI database are underlined (see Data Set S2).
[b]All strains of *K. oxytoca* complex also have intrinsic bla_{OXY} genes.

In conclusion, the O- and K-antigen types of *K. oxytoca* complex overlap those of *K. pneumoniae*. Although several STs appear to be relatively common, no internationally distributed high-risk clones associated with the spread of carbapenem resistance were identified at present. Among the 9 species of *K. oxytoca* complex, *K. oxytoca* and *K. michiganensis* appear to be the major ones seen in human infections, which could be associated with the relatively more virulence factors that they harbor. Although the information generated by analysis of currently available genomes is helpful, due to the bias of sampling for genome sequencing, well-designed surveillance studies are required to provide insights in the prevalence, pathogenesis, antimicrobial resistance, and spread of *K. oxytoca* complex.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.5 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 5, XLSX file, 0.02 MB.

ACKNOWLEDGMENTS

The relevant works of the authors were supported by the National Natural Science Foundation of China (81861138055) and by a grant from the West China Hospital of Sichuan University (1.3.5 project for disciplines of excellence, grant ZYYC08006).

We declare there are no conflicts of interest.

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